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Ultrastructural Localization of Antho-RWamides I and II at Neuromuscular Synapses in the Gastrodermis and Oral Sphincter Muscle of the Sea Anemone *Calliactis parasitica*

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Abstract. Light microscopic studies have shown that the sea anemone neuropeptides Antho-RWamides I (<Glu-Ser-Leu-Arg-Trp-NH_2) and II (<Glu-Gly-Leu-Arg-Trp-NH_2) are located in neurons associated with the oral sphincter muscle of the sea anemone *Calliactis parasitica*. In the present ultrastructural study, using the immunogold technique, we found Antho-RWamide-like material in the granular vesicles of neurons that make synaptic contacts with the myonemes of both gastrodermal and oral sphincter muscle cells of *Calliactis*. Gastrodermal nerve cells contained immunoreactive granular vesicles averaging 149.3 ± 4.1 nm in diameter; smaller granular vesicles (47.5 ± 2.5 nm) were present at a labelled synapse. Neurites associated with the sphincter muscle had immunoreactive granular vesicles averaging 78.8 ± 3.3 nm in diameter with smaller granular vesicles (63 ± 4.4 nm) at three labelled neuromuscular synapses. All Antho-RWamide-immunoreactive vesicles were irregularly granular, unlike the typical dense-cored vesicles observed at some other synapses in sea anemones. No evidence was found of storage or release at nonsynaptic sites (paracrine secretion).

The Antho-RWamide immunoreactive neurites innervate the sphincter muscle fibers directly rather than through intermediate neuronal pathways. This is the first ultrastructural evidence of a neuropeptide at a coelenterate neuromuscular synapse.

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Abbreviations. BSA, bovine serum albumin; PBS, phosphate-buffered saline.

Introduction

Nervous systems first appeared in cnidarians or in a closely related ancestor group. The basic plan of the cnidarian nervous system is a diffuse network of nerve cells, but in some members of this group, such as medusae, nerve cells also can aggregate in nerve plexuses, nerve rings, or sense organs. Sea anemones have complex neuronal nets and nerve plexuses in both the inner and outer epithelial layers (Grimmelikhuijzen and Westfall, 1995). From sea anemones, a variety of neuropeptides, including the closely related Antho-RWamide I (<Glu-Ser-Leu-Arg-Trp-NH_2) and Antho-RWamide II (<Glu-Gly-Leu-Arg-Trp-NH_2) have been isolated (Graff and Grimmelikhuijzen, 1988a, b; Grimmelikhuijzen *et al.*, 1992). The Antho-RWamides are present in neurons of many body regions of sea anemones, but Antho-RWamide-immunoreactive neurons are especially dense in the upper body column, where they innervate the oral sphincter muscle (Graff and Grimmelikhuijzen, 1988a; Grimmelikhuijzen *et al.*, 1989, 1992). The oral sphincter muscle is a ring of circular muscle fibers embedded in the gelatinous middle layer, the mesoglea, of the upper body wall. During periods of danger and environmental stress, it contracts to close the animal and protect the retracted apical tentacles. The cell bodies of the Antho-RWamide-positive neurons innervating the sphincter appear to be located in the gastrodermis (endoderm) of the upper body wall, whereas their processes project across the mesoglea and ramify into long, fine projections paralleling the circular bundles of sphincter muscle fibers (Graff and Grimmelikhuijzen,

1988a; Grimmelikhuijzen *et al.*, 1989, 1992). No synaptic contacts between neurons and muscle fibers can be seen at the light microscope level.

In physiological experiments, the Antho-RWamides (10^{-8} *M*) induced tonic contractions in isolated oral sphincter muscle rings and cells isolated from the sphincter (McFarlane *et al.*, 1991). Taken together, these data indicate that the Antho-RWamides are transmitters at neuromuscular synapses.

Electron microscopic "immunogold" techniques, using neuropeptide antisera and colloidal gold-conjugated secondary antibodies, have permitted the ultrastructural localization of neuropeptides in dense-cored or granular vesicles of a variety of cnidarian neurons (Koizumi *et al.*, 1989; Singla and Mackie, 1991; Westfall and Grimmelikhuijzen, 1993). Antho-RFamide (<Glu-Gly-Arg-Phe-NH_2), the first sea anemone neuropeptide to be isolated, was demonstrated in dense-cored vesicles of bidirectional, interneuronal synapses of sea anemones (Westfall and Grimmelikhuijzen, 1993). In the present study, using the immunogold technique with an antiserum against the common C terminus of the Antho-RWamides, we were able to label granular synaptic vesicles at neuromuscular junctions of sea anemones. This strongly supports our hypothesis that the Antho-RWamides are transmitters at some cnidarian neuromuscular synapses.

Materials and Methods

Three specimens of the sea anemone *Calliactis parasitica* (sent from Roscoff Station Biologique, France) were anesthetized using 0.3 *M* $MgCl_2$. Once relaxation was sufficient, the animals were cut using Personna Gem super stainless steel blades.

For light microscopy, one animal was placed in 2.5% glutaraldehyde in 0.05 *M* sodium cacodylate, pH 7.4, and cut longitudinally in half. Photographs were taken using an OM-2S Olympus camera attached to a Wild model M75 zoom stereomicroscope to locate the sphincter muscle. Longitudinal slices of the oral sphincter from the other half of the animal were processed, embedded in paraffin, sectioned, mounted on glass slides, and stained with haematoxylin and eosin. Photographs were taken of the sphincter muscle using an Aristoplan image analysis light microscope.

For electron microscopy, two animals were cut longitudinally, and the lower body columns removed. Several longitudinal slices were cut, starting at one edge and proceeding serially. Each slice contained a few tentacles. The slices were placed in one of two fixatives: 4% paraformaldehyde—0.1% glutaraldehyde in 0.1 *M* phosphate buffer, pH 7.4; 4% paraformaldehyde—0.1% glutaraldehyde in 0.1 *M* phosphate buffered saline, pH 6.5 for 30 min, and then pH 11.0 for 3 h (Berod *et al.*, 1981).

All tissues were rinsed in 0.1 *M* phosphate buffer, pH 7.4, dehydrated in ethanol, and then in acetone; infiltrated overnight in a mixture of Epon and Araldite; and cut into small segments for final embedding by taking horizontal slices down the length of the oral sphincter starting at the region near the tentacles.

Thin longitudinal sections of sphincter muscle were cut with a diamond knife and mounted on Formvar-coated, 100-mesh, nickel grids. The sections were rinsed in doubly distilled water (ddH_2O), then exposed to saturated sodium metaperiodate for 30 min to open antigenic sites. After a ddH_2O rinse, the sections were exposed to normal goat serum diluted 1:20 with PBS-Tween-BSA buffer to block nonspecific antigenic sites. They were incubated for 1 h with rabbit antiserum #2061 against Antho-RWamide, diluted 1:50–1:200 with buffer.

After rinsing in buffer, the sections were immunogold stained for 1 h in goat anti-rabbit IgG conjugated to either 5 or 15 nm-gold particles, diluted in buffer 1:10–1:40. After rinsing in buffer with BSA, then in PBS, they were postfixed for 15 min in 2% glutaraldehyde in PBS and rinsed in ddH_2O. The sections were further stained in 7% uranyl acetate in 70% ethanol, then in Reynolds lead citrate and examined in a Philips 400 transmission electron microscope. Because only 2–3 sections covered a grid, and usually 10 grids were used per experiment, the search for synapses was slow and laborious.

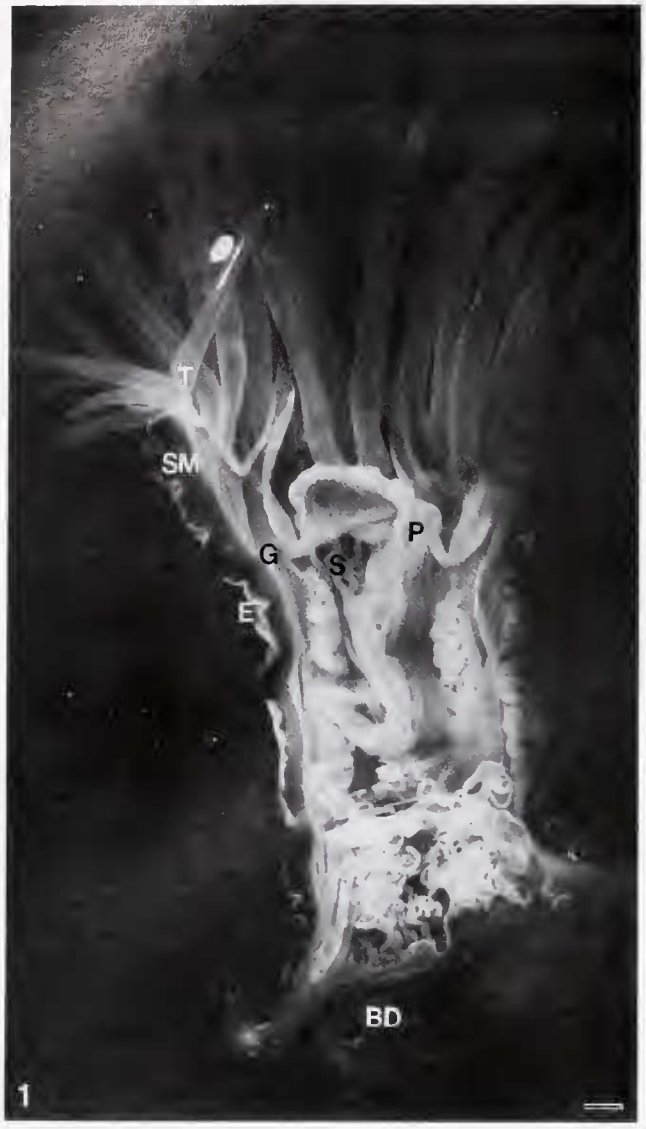
Control sections were exposed to Antho-RWamide antiserum (1:200), which had been incubated overnight in 100 μg/ml of Antho-RWamide.

Antiserum #2061 directed against the C terminus (Arg-Trp-NH_2) of both Antho-RWamides I and II was prepared as described by Grimmelikhuijzen (1985). Arg-Trp-NH_2 was a customer synthesis by Bachem (Bubendorf, Switzerland). Only antisera against Arg-Trp-NH_2 and no other antisera against the other sea anemone Arg-X-NH_2 peptides stained neurons in the sphincter muscle (see *e.g.*, Fig. 2 of Grimmelikhuijzen *et al.*, 1992).

To categorize a granule type, measurements were made of 10 randomly selected granules or vesicles per gastrodermal neuron or sphincter muscle neurite, and four to five granules or vesicles per synapse. The reason why only granules were measured in some cases is owing to the fact that the two-pH paraformaldehyde fixation, which worked best for immunogold labeling with antisera to the Antho-RWamides, caused some loss of membrane preservation around many granules.

Results

The oral sphincter muscle of *Calliactis parasitica* was located in a widened region of the upper body column mesoglea (Fig. 1). It was composed of multiple layers of myonemes forming the circular, smooth, muscle fibers



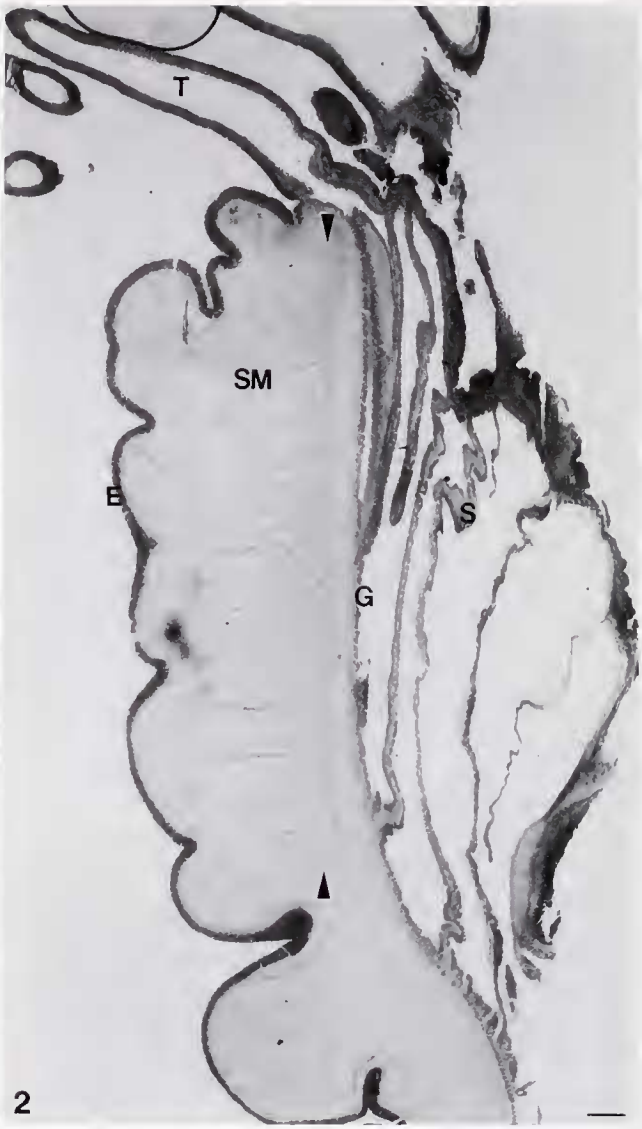


Figure 1. Longitudinal section through whole glutaraldehyde-fixed specimen of *Calliactis parasitica* indicating thickened mesoglea containing oral sphincter muscle (SM) at base of tentacles (T). Note epidermis (E), gastrodermis (G), septa (S), pharynx (P), and basal disk (BD). Bar = 1000 μm.

Figure 2. Light micrograph of a cross section through the oral sphincter muscle (SM). Note longitudinal section of tentacle (T), epidermis (E), diffuse bundles of myonemes (between arrowheads) within the mesoglea, gastrodermis (G), and septa (S). Bar = 100 μm.

encompassing the oral region. In a longitudinal section of the animal, the bundles of oral sphincter myonemes extended one tenth of the length of a 3-cm-long sea anemone and appeared to increase in number near the oral region (Fig. 2). The myonemes extended irregularly towards the epidermis, but stopped abruptly near a band of mesoglea separating them from the gastrodermis.

Using electron microscopy, we observed various-sized granular vesicles in bipolar-like nerve cells of the gastrodermal nerve net. The granules, which averaged 149.3 ± 4.1 nm in diameter, were immunoreactive to Antho-RWamide (Fig. 3). The granules labeled with both 5 nm gold (upper inset Fig. 3) and 15 nm gold (middle inset) and were present at a neuromuscular synapse (lower inset). The synaptic vesicles averaged 47.5 ± 2.5 nm in diameter.

Nerve processes from the gastrodermis crossed the muscle-free border of the mesoglea and entered into the individual oblong bundles of oral sphincter myonemes (Fig. 4). These myonemes were composed of closely packed bundles of myofilaments, aggregated at one side, and of a myofilament-free area at the other side. An

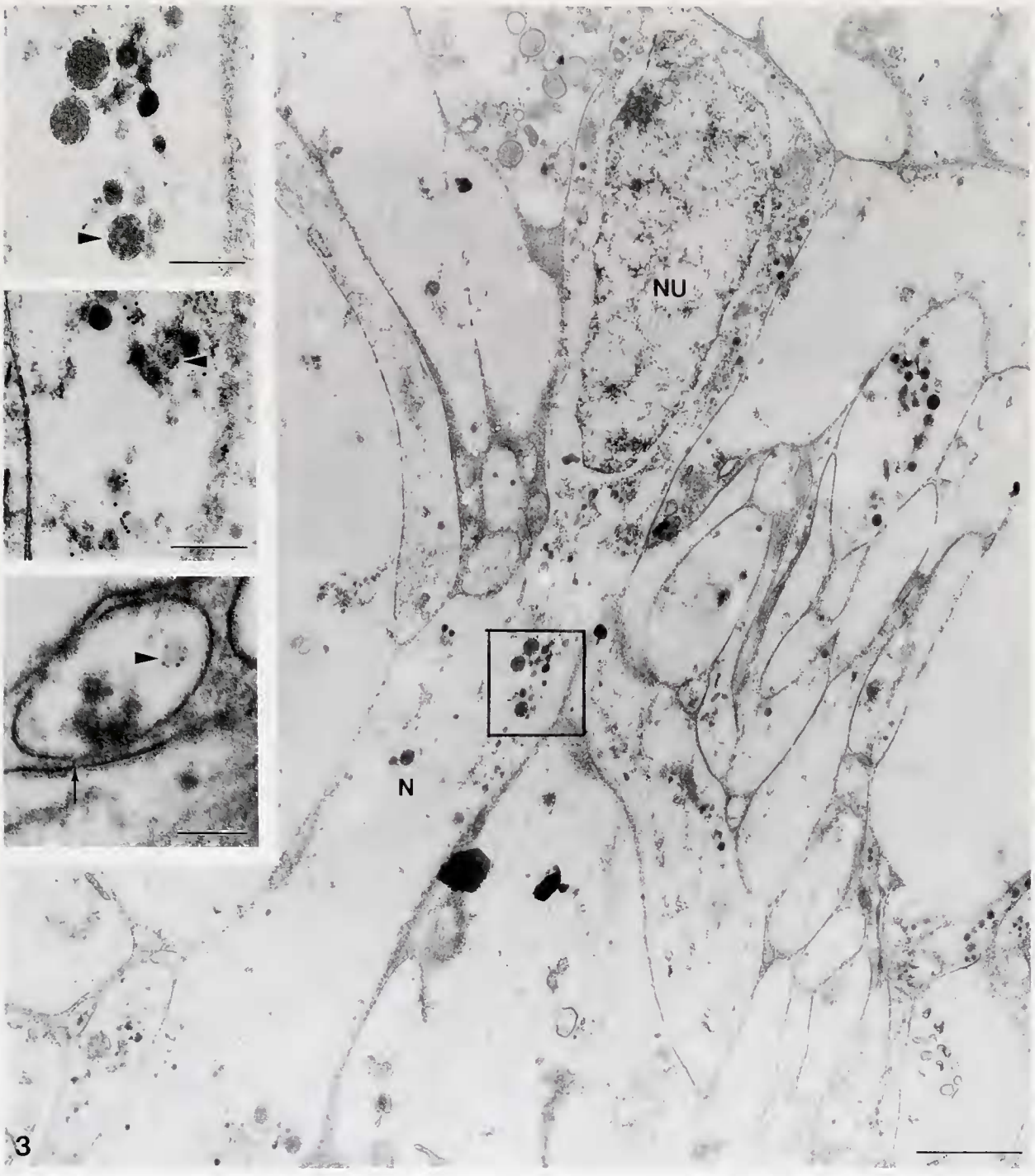


Figure 3. Electron micrograph of neuronal perikaryon and neurite containing granular vesicles immunogold-labeled with antisera to Antho-RWamide in gastrodermal nerve plexus. Note large neurite (N) with various-sized granular vesicles (box) and nucleus (NU) of neuron. Bar = 1 μm. Insets: High magnification of boxed area of neurite with 5 nm gold marker in large granules (arrowhead, upper inset), serial section of neurite with smaller granules labeled with 15 nm gold particles (arrowhead, middle inset), and 5 nm gold particles in granular vesicles (arrowhead, lower inset) at a neuromuscular synapse with transverse filaments in the synaptic cleft (arrow). Bar = 0.25 μm (upper; middle) and 0.1 μm (lower).

Figure 4. Ultrathin section of Antho-RWamide immunoreactive neurite (N) passing into a bundle of oral sphincter myonemes (MY) in the mesoglea (ME). Note small neurites with either clear or granular vesicles (arrowheads). Bar = 1 μm. Inset: High magnification of neurite with immunogold-labeled granular vesicles. Bar = 0.25 μm.

occasional nucleus was observed in these myonemes, suggesting that they are complete muscle cells. The neurites that invaded the sphincter usually were associated with the myofilament-free areas of the myonemes.

Dense, granular vesicles, varying in size and having an average diameter of 78.8 ± 3.3 nm were distributed unequally within the slender, 0.2–0.3 μm-diameter neurites. Groups of these granules were immunoreactive to Antho-RWamide antisera using both 5 and 15 nm gold markers (inset Fig. 4). Most neurites observed within individual bundles of myonemes had densely granular vesicles, although occasional groups of nongranular vesicles were present.

Typical neuromuscular synaptic foci were few in number and difficult to locate in the oral sphincter muscle, but several putative immunoreactive neuromuscular synapses were observed (Fig. 5). The presynaptic vesicles were aligned at the presynaptic membrane opposite a series of cross filaments in the synaptic cleft and a postsynaptic density (Fig. 5b). The synaptic cleft ranged from 9 to 18 nm in width. Sometimes, it took two-to-three serial sections through a synapse to verify the presence of cross filaments in the synaptic cleft at loci where granular vesicles were gold-labeled with antisera to Antho-RWamide. Occasionally, vesicles attached to the presynaptic membrane appeared empty (Fig. 5b), although gold label was present. The synaptic vesicles averaged 63 ± 4.4 nm in diameter.

Experimental serial sections, incubated in Antho-RWamide antisera, had immunoreactive granular vesicles in some neurites (Fig. 6a, b). Control sections, incubated in antisera which had been incubated overnight in 100 μg/ml of Antho-RWamide, did not stain with immunogold (Fig. 6c). A neurite adjacent to those with immunoreactive granular vesicles contained electron-lucent vesicles, which were not immunoreactive to Antho-RWamide antisera (Fig. 6a, b).

Discussion

Both Antho-RWamides I and II stimulate contractions in rings of sphincter muscle and in isolated sphincter muscle cells from *Calliactis parasitica* (McFarlane *et al.*, 1991). In this study, we have found that neurons make

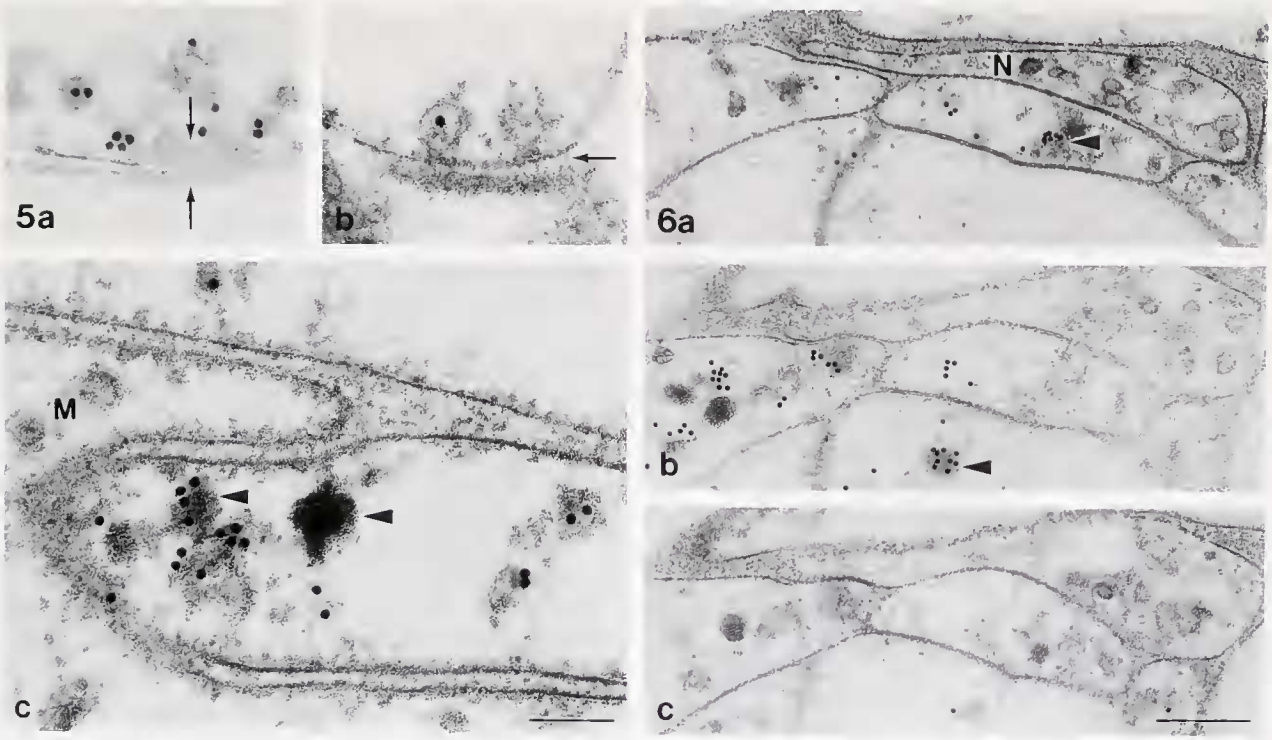


Figure 5. Three examples (a, b, c) of neuromuscular synapses with Antho-RWamide-labelled presynaptic granules and vesicles in oral sphincter muscle. Note parallel pre- and postsynaptic densities (between arrows, a), cross filaments in synaptic cleft (arrow, b), and 15 nm-gold label specific to presynaptic granular vesicles (arrowheads, c). Muscle cell (M). Bar = 0.1 μm.

Figure 6. Three serial thin sections through a cross-sectioned bundle of oral sphincter myonemes revealing immunoreactive granular vesicles in experimental (a,b) and nonimmunoreactive granular vesicles in control section (c). Note clear vesicles without gold label in adjacent upper neurite (N). Bar = 0.25 μm.

morphologically identifiable synapses with the sphincter muscle cells and that these synapses contain Antho-RWamide-immunoreactive granular vesicles. This supports our hypothesis that the Antho-RWamides are transmitters at the neuromuscular junctions of the sphincter.

In a previous study, we located Antho-RFamide immunoreactivity in granular vesicles at two-way interneuronal synapses in the sea anemone *Anthopleura* (Westfall and Grimmelikhuijzen, 1993). Therefore, peptidergic synapses clearly play an important role in primitive nervous systems.

The long slender neurites, which cross the mesoglea to innervate the sphincter muscle, contain Antho-RWamide immunoreactive granules of varying sizes, whereas the granular vesicles at synapses appear to be somewhat smaller. The synthesis of vertebrate neuropeptides follows a stepwise pattern: a prepropeptide is formed in the rough endoplasmic reticulum, then reduced in the Golgi complex to a smaller propeptide and is eventually cleaved into the active peptides in vesicles (Brownstein, 1982). This pattern of synthesis and reduction of the Antho-RWamide precursor may also occur in sea anemone neurons, and it may explain the difference in size between granules in the neurites and in the synapses. Similarly, an immunoreactive nucleated nerve cell in the gastrodermal nerve plexus has both large and small granular vesicles, the latter being denser. Slightly smaller granular vesicles have been observed at an immunoreactive gastrodermal neuromuscular synapse.

Sea anemone muscles undergo spontaneous autonomous movements similar to those of the smooth muscle of the vertebrate intestine, which result in constriction and extension of this tube-like structure (Parker, 1919). Autonomic nerve fibers, which innervate smooth muscle of the vertebrate intestine, do not always terminate with morphologically distinct neuromuscular synapses (Jänig, 1978). A similar situation might exist in the cnidarian smooth muscles. However, some neuromuscular junctions do occur in cnidarians and can be recognized by the presence of dense-cored or clear vesicles (80–100 nm in diameter) aligned on the presynaptic side of a pair of electron-dense synaptic membranes separated by a 15–25 nm-wide cleft (Westfall, 1973).

In this study of gastrodermal and oral sphincter neuromuscular synapses of the sea anemone *Calliactis parasitica*, there are two to four or more granular vesicles aligned at paired, electron-dense, synaptic membranes separated by a 15-nm-wide cleft traversed by a series of cross filaments. The neuromuscular synapses are similar ultrastructurally to the interneuronal synapses in the sea anemone nerve plexus (Westfall, 1970, 1987). Electron-lucent vesicles, which do not label with antisera to Antho-RWamide, are present in a few other neurites. Although nothing is known about the putative neurotransmitter substances at sea anemone neuromuscular synapses with electron-lucent vesicles, this study demonstrates Antho-RWamide immunoreactivity in granular vesicles at neuromuscular synapses.

Ross (1960a,b) has stated that adrenaline causes contraction in sphincter and circular muscle preparations of *Calliactis parasitica* and *Metridium senile;* Wood and Lentz (1964) have claimed that adrenaline is present in the mesenteries of *Metridium*. Other studies have suggested that catecholamines (Dahl *et al.*, 1963; Anctil *et al.*, 1984; DeWaele *et al.*, 1987; Umbriaco *et al.*, 1990) and DOPA (Carlberg, 1983) are present in nerve cells of various anthozoa. Taurine-like immunoreactivity has been reported in the motor nerve net of the scyphozoan jellyfish *Cyanea capillata* (Carlberg *et al.*, 1995). Thus, besides Antho-RWamides, other neuromuscular transmitters also may be present in the anthozoans.

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